

Cell Reports, Volume 16

Supplemental Information

RNF4-Dependent Oncogene Activation

by Protein Stabilization

Jane J. Thomas, Mona Abed, Julian Heuberger, Rostislav Novak, Yaniv Zohar, Angela P. Beltran Lopez, Julie S. Trausch-Azar, Ma. Xenia G. Ilagan, David Benhamou, Gunnar Dittmar, Raphael Kopan, Walter Birchmeier, Alan L. Schwartz, and Amir Orian

Thomas *et al.* Supplemental Information (SI):

1. Supplemental Figures and Legends (S1-S6).

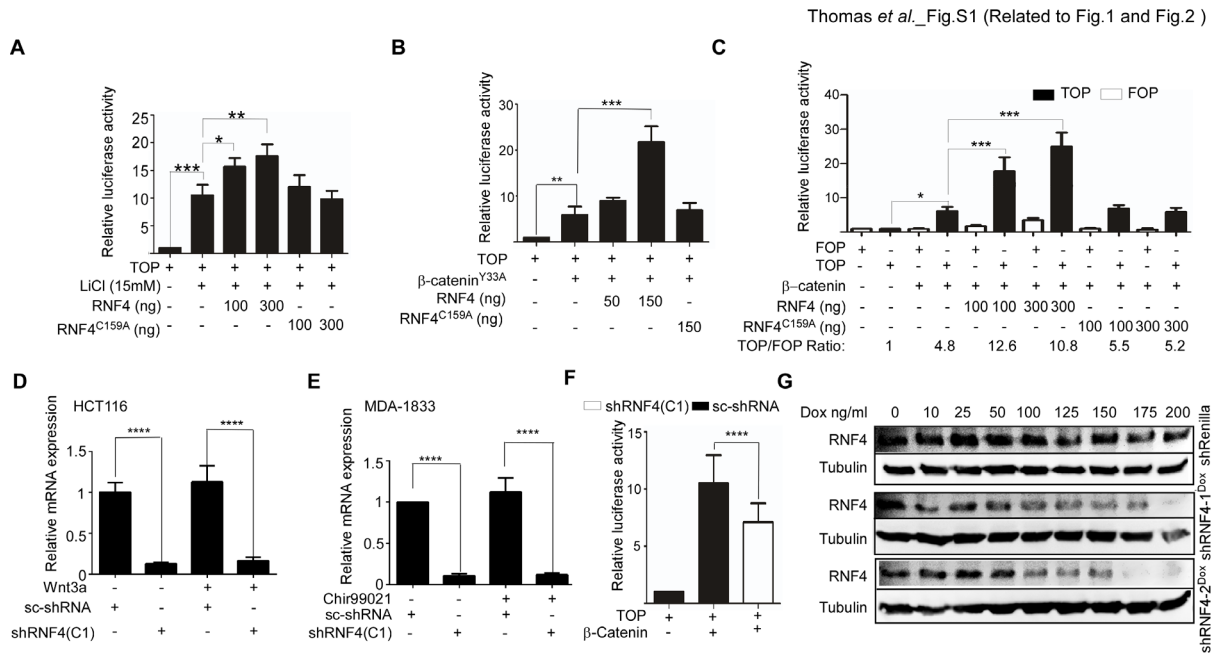


Figure S1 (Related to Figs. 1 and 2): *RNF4* mRNA levels and its impact on Wnt pathway transcriptional activation.

(A-C) TOP-reporter luciferase assays in HEK293T cells. Data shown are mean ± SE from three independent experiments performed in triplicate unless otherwise stated. (A) LiCl-induced activation of the TOP reporter is enhanced by increasing amounts of RNF4 but not RNF4^{C159A}. (B) Increasing amounts of RNF4 but not RNF4^{C159A} enhance the transcriptional activity of the oncogenic β-catenin^{Y33A} mutant (n=4). (C) RNF4 enhancement of transcription requires intact TRE elements. RNF4 does not enhance transcription from the FOP control reporter, which harbors mutant TCF binding sites. (D) qPCR analysis of *RNF4* mRNA levels in HCT116 cells without or after 4h exposure to 40ng/ml Wnt3a. (E) Analysis similar to (D) of MDA1833 cells targeted by constitutive control (“sc”) or RNF4-shRNA, with or without 2.5μM Chir92201 treatment. Data shown are mean ± SE from three independent experiments. (F) β-catenin-induced activation of the TOP luciferase reporter is modestly reduced in HEK293T cells infected with RNF4-shRNA but not with control sc-shRNA (n=8, **** P<000.1). (G) Western blot analysis of RNF4 protein levels in SW620 cells expressing Dox-induced control (Renilla), and two independent shRNA targeting RNF4 (shRNF4-1^{Dox}, shRNF4-2^{Dox}) in the presence of increasing Dox concentrations.

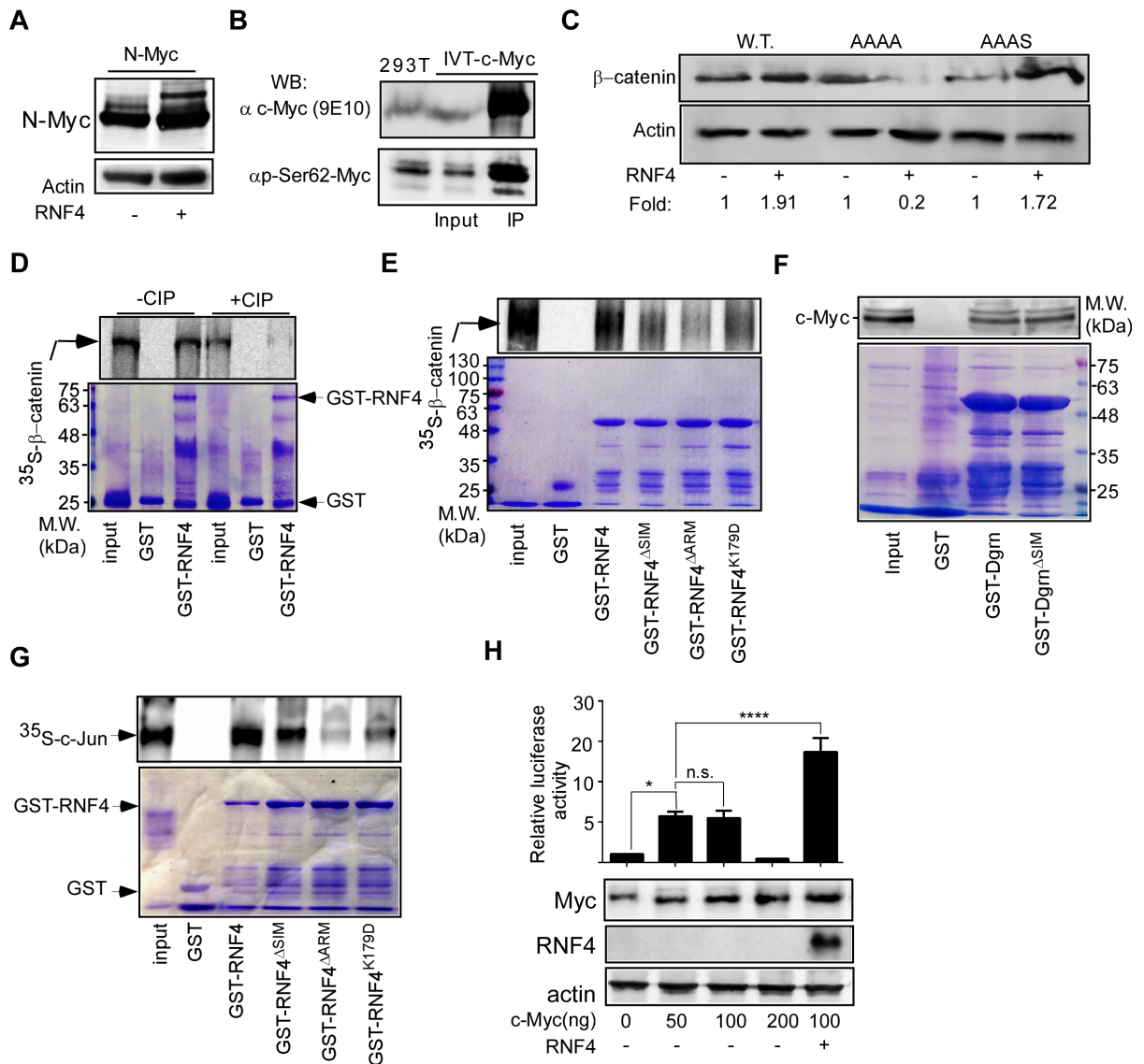


Figure S2 (Related to Figs. 2 and 4): Mechanisms involved in RNF4-dependent ubiquitylation and stabilization.

(A) Expression of RNF4 in HEK293T cells increases steady-state N-Myc levels. (B) Ser62 c-Myc is phosphorylated in the TNT-IVT system. Western blot analyses of reticulocyte-TNT-IVT c-Myc protein using c-Myc (9E10) and p-Ser62-c-Myc antibodies. Extract derived from HEK293T cells expressing c-Myc serves as positive control. Input is 10% of IP material. α -cMyc antibody (SC#789 1:200) was used for IVT-c-Myc IP, and c-Myc was detected using the indicated c-Myc antibodies. (C) FLAG- β -catenin, and FLAG- β -catenin^{AAAS}, but not FLAG- β -catenin^{AAAA} are stabilized by RNF4 in HEK293T cells expressing the indicated plasmids (β -catenin^{AAAA} is a β -catenin mutant where Ser³³, Ser³⁷, Thr⁴¹, and Ser⁴⁵ were replaced with Ala residues. β -catenin^{AAAS} is a β -catenin mutant where Ser³³, Ser³⁷, Thr⁴¹, were replaced with Ala and Ser⁴⁵ is intact). (D) Mock-treated but not CIP-treated ³⁵S-Met- β -catenin binds to GST-RNF4 (CIP, Calf Intestinal Phosphatase). (E) *In vitro* translated (IVT) ³⁵S-Met- β -catenin binds well to RNF4, modestly to dRNF4^{ΔSIM} and RNF4^{K179D}, but only minimally to GST-RNF4^{ΔARM}. (F) *In vitro* translated (IVT) c-Myc binds to GST-Dgrn (dRNF4), and to GST-Dgrn^{ΔSIM} (G) ³⁵S-Met-c-Jun binds well to RNF4, modestly to dRNF4^{ΔSIM} and RNF4^{K179D}, but only minimally to RNF4^{ΔARM}. In D-G lower panels are coomassie gels respectively, and 5% input is shown. (H) Myc-luciferase reporter assays in HEK293T cells transfected with the indicated plasmids. Lower panels are western blot analyses of steady-state levels of c-Myc, and RNF4 (n=3, Data shown are mean \pm SE from three independent experiments).

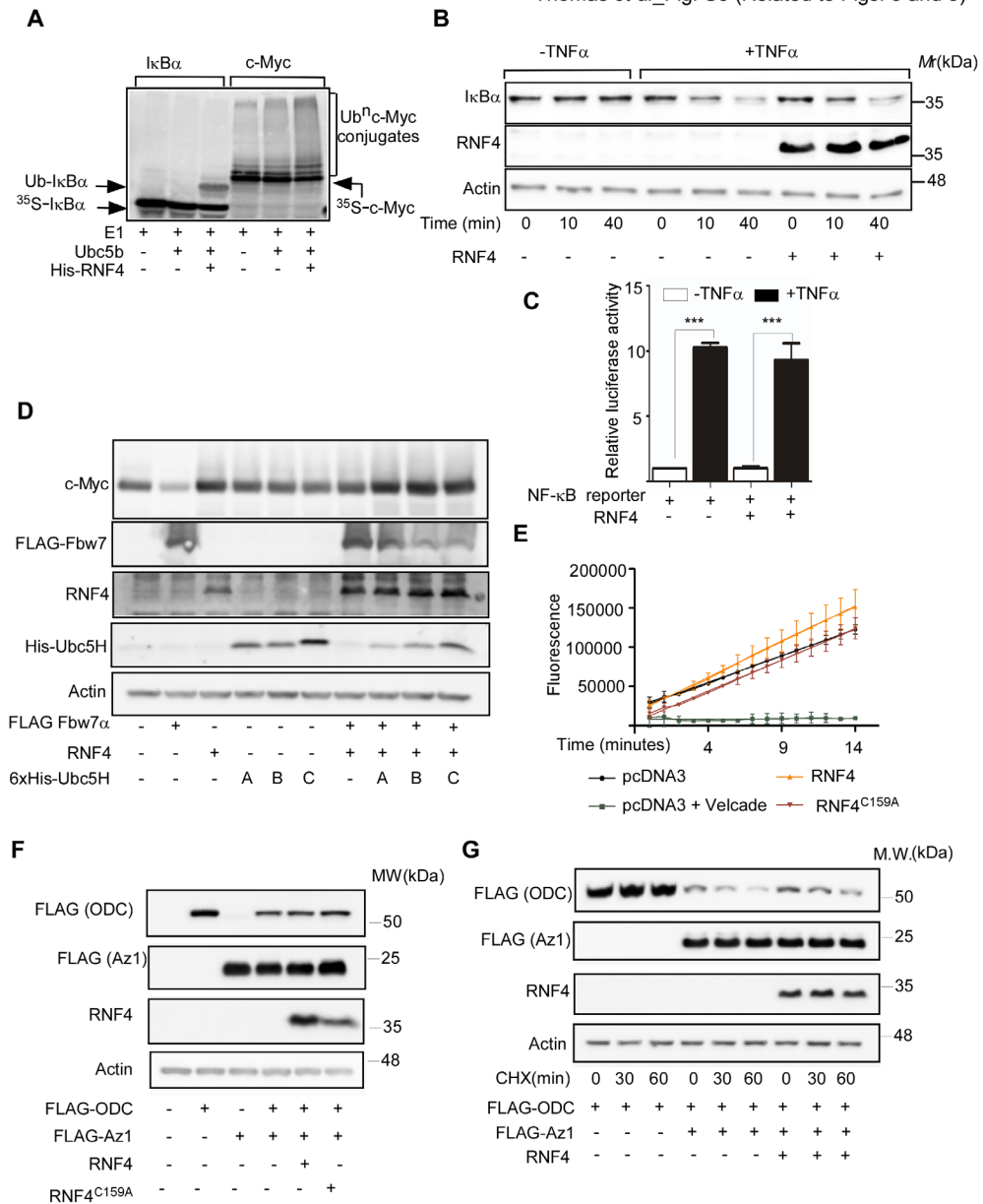


Figure S3 (Related to Figs. 3 and 5): RNF4 activity is selective, is not alleviated by co-expression of UbcH5 enzyme, and does not impair 26S proteasomal activity.

(A) *In vitro* ubiquitylation of ³⁵S-Met-c-Myc or ³⁵S-Met-I κ B α using purified E1, Ubc5Hb and RNF4 in a partially reconstituted system. “Ubⁿ-Myc Conjugates” denotes high molecular weight c-Myc~poly-ubiquitin conjugates. (B) TNF α -induced degradation of I κ B α , is not affected by RNF4 expression. Cells were transfected with indicated plasmids and the protein levels of I κ B α and RNF4 were determined after TNF α or mock treatment for the indicated time. Actin served as loading control. (C) TNF α -induced activation of an NF κ B-luciferase reporter is not altered by expression of RNF4. HEK293T cells were transfected with indicated plasmids and 48h later cells were treated with TNF α as indicated. Subsequently, NF κ B-Luciferase reporter activity was determined. Data shown are mean \pm SE from three independent experiments. (D) RNF4-dependent c-Myc stabilization is not alleviated by co-expression of UbcH5 ubiquitin conjugating enzyme (E2) isoforms alone or in the context of SCF^{Fbw7} expression. HEK293T were transfected with indicated proteins, and 48 hours later cell lysates were analyzed for indicated proteins by western-blot analysis. Actin served as loading control. (E) Proteasomal catalytic activity in HEK293T-derived lysates is not altered by expression of RNF4 (orange) or RNF4^{C159A} (brown). However, pre-incubation of lysates with the proteasomal inhibitor Velcade[®] (green) blocked ubiquitin-independent proteasomal degradation of the LLVY peptid (Gillette et al., 2008). (F-G) Ubiquitin-independent, Az1-induced proteasomal degradation of ODC (Bercovich, et al., 1989), is unaffected by expression of RNF4 or RNF4^{C159A}. (F) Steady-state protein levels of exogenous ODC decrease in HEK293T cells overexpressing Az1, an effect not alleviated by RNF4 or RNF4^{C159A} expression. (G) Dynamic CHX-chase experiment in HEK293T cells expressing indicated plasmids encoding ODC, Az1, and hRNF4, followed by western analysis of ODC protein levels. Actin served as loading control.

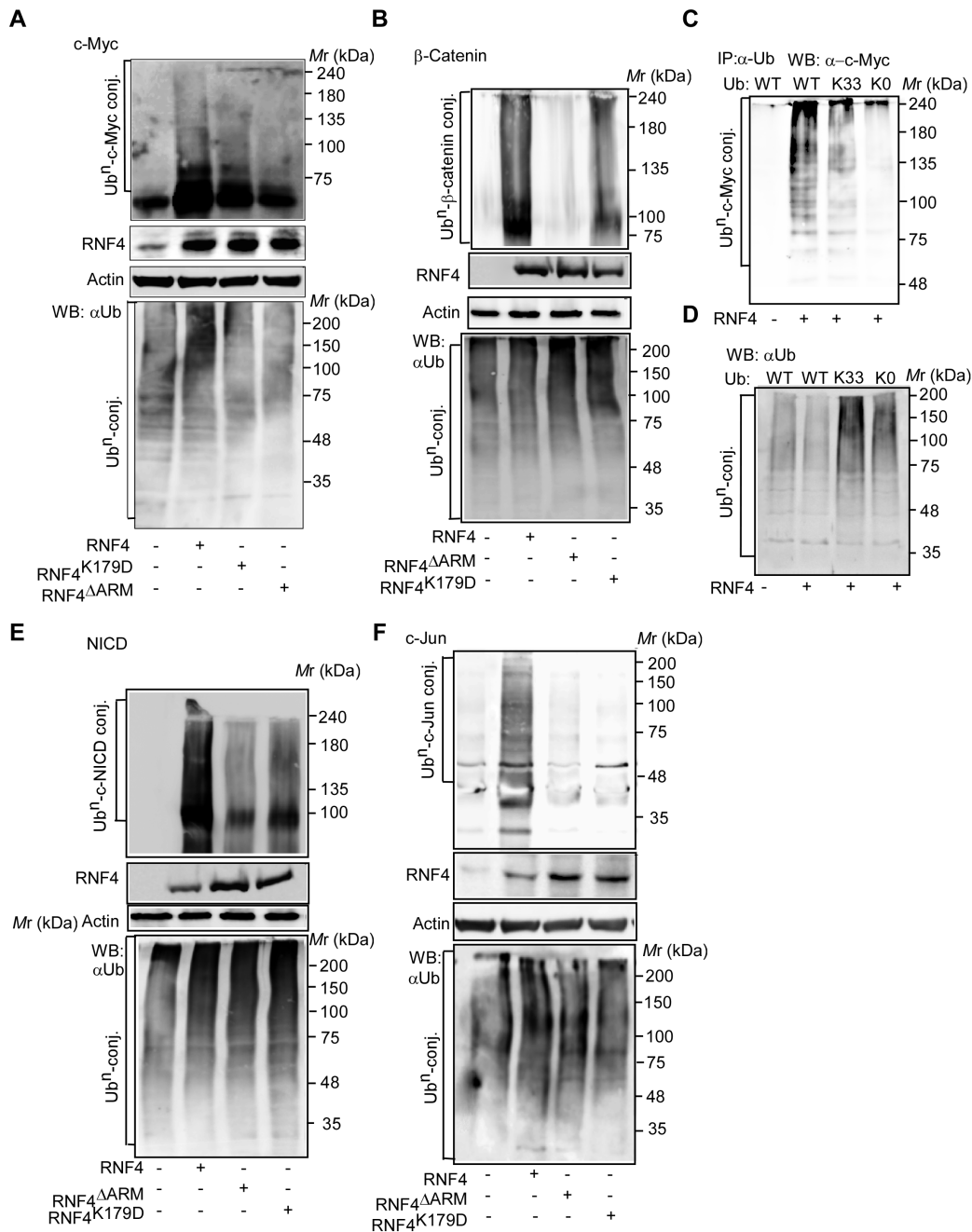


Figure S4 (Related to Figs. 4, 5, and 6): Requirements for RNF4-dependent ubiquitylation.

(A, B) Upper panels: Ubiquitylation of c-Myc, and FLAG- β -catenin in HEK293T cells expressing c-Myc (A), FLAG- β -catenin (B), 6His-Ub, and where indicated plasmids coding either GFP (control), RNF4, RNF4^{K179D}, or RNF4 ^{Δ ARM}. Lower panels: Overall ubiquitylation determined using an α -Ub antibody. (C) RNF4-dependent poly-ubiquitylation of c-Myc requires K33 within ubiquitin. Upper panel: c-Myc ubiquitylation was performed in HEK293T cells transfected by HA-ubiquitin or indicated K>R ubiquitin mutants. c-Myc-ubiquitin conjugates were recovered using α HA-beads and ubiquitylated-Myc forms were detected using anti c-Myc antibody (9E10). (D) Overall ubiquitylation determined using an α -Ub antibody. (E-F) Upper panels: ubiquitylation in HEK293T cells expressing, FLAG-NICD (E), c-Jun (F). Cells were co-transfected with plasmids coding for either GFP (control), RNF4, or RNF4 ^{Δ ARM}, or RNF4^{K179D}. Ubiquitylation was performed similar to Fig. 5A, and the protein level of RNF4 and RNF4 mutants is shown. Actin serves as a loading control. Lower panels: Overall ubiquitylation was determined using an α -Ub antibody.

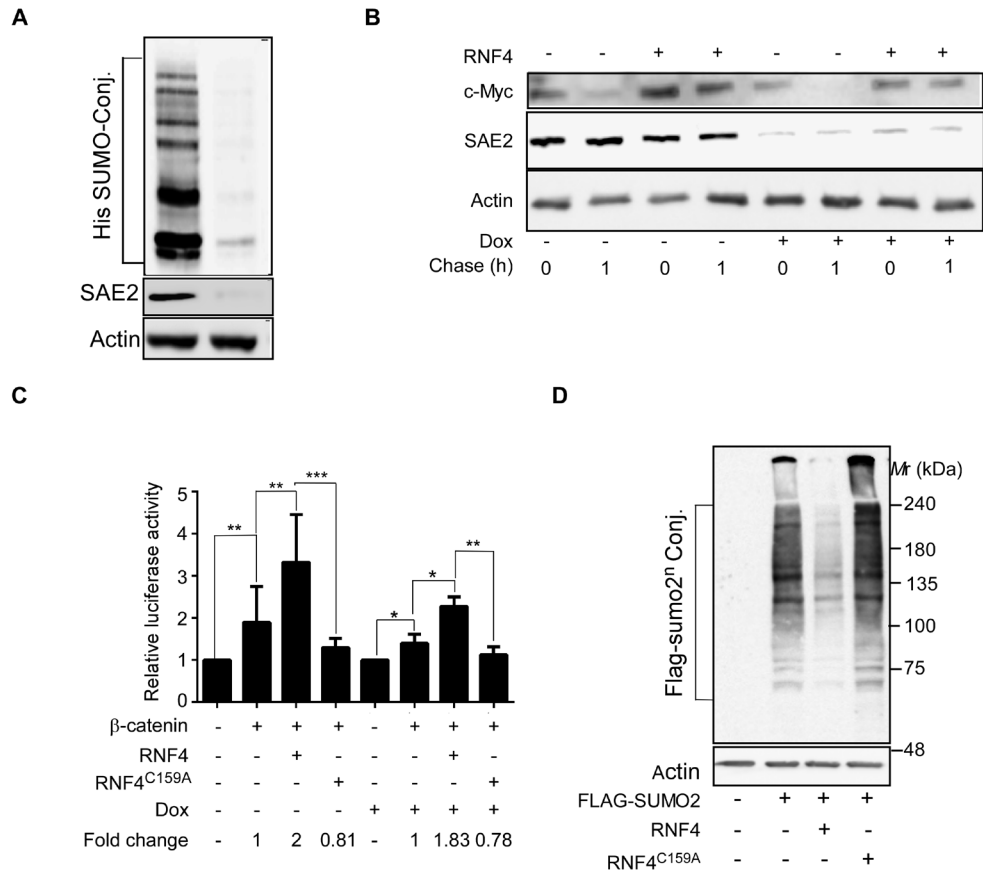


Figure S5 (Related to Fig. 6): RNF4-dependent protein stabilization and transcriptional activation are independent of de-novo SUMOylation.

(A) Western-blot analysis of indicated proteins in control and Dox-treated MDA-MB231^{shSAE2Dox} cells transfected with plasmid coding for His-SUMO2. SUMOylated proteins were recovered by Ni-NTA beads and visualized using α -SUMO2 antibody. (B) Western-blot analysis of HA-c-Myc and endogenous SAE2 protein levels in dynamic CHX chase experiments in MDA-MB231^{shSAE2Dox} cells. Cells were transfected with indicated plasmids and either GFP (“-“), or RNF4 in the presence or absence of doxycycline (Dox). Lysates were run on SDS-PAGE and blotted with indicated antibodies. Actin serves as loading control. (C) TOP-reporter luciferase assays with indicated plasmids in MDA-MB231^{shSAE2Dox} cells, expressing RNF4 or RNF4^{C159A} in the presence or absence of Dox (n=4, Data shown are mean \pm SE from four independent experiments performed in triplicates). (D) Expression of RNF4 but not mutant RNF4^{C159A} decreases levels of SUMOylated proteins. HEK293T cells were transfected with indicated plasmids and lysed 48h later in denaturing buffer. SUMO[n]-Conj. denotes poly-SUMOylated conjugated proteins.

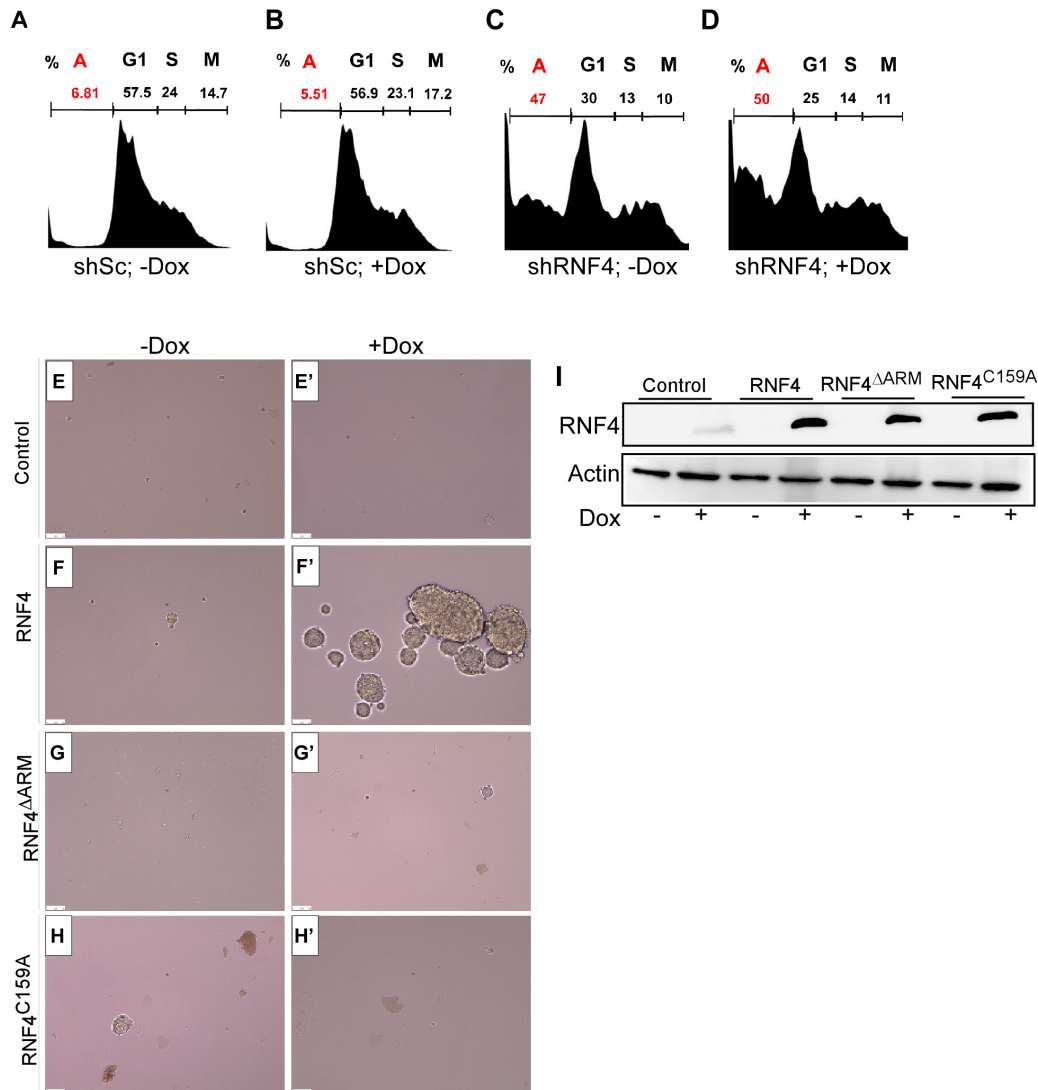


Figure S6 (Related to Fig. 7): RNF4 is required for cancer cell survival and its expression enhances sphere formation of SW480 colon cancer cells. (A-D) Analysis of cell cycle phasing using FACS of control (sc-shRNA), or shRNF4(C1) expressing cells. (A-B) Targeting with sc-shRNA, control. (C-D) Targeting with constitutive shRNF4(C1). (A, C) Targeting of sc-shRNA or shRNF4(C1) in the presence of SAE2, (Dox-). (B, D) Targeting of sc-shRNA control or shRNF4(C1) upon SAE2 inactivation (Dox+). (E-H') RNF4 enhances the ability of SW480 colon cancer cells to form spheres in non-adherent medium. SW480 cells infected with lentivirus coding for Dox-induced control, RNF4, or RNF4 mutants were seeded in the absence (E-H) or presence of 200ng/ml Dox (E'-H') and, sphere formation was evaluated one week after seeding. (I) Western blot analyses of RNF4 protein level in SW480 colon cancer cells infected with the indicated viruses in the absence or presence of 200ng/ml Dox.

2. Supplemental Experimental Procedures:

Materials: MG132 was from Boston Biochemicals, and Valcade® was a kind gift from Ariel Stanhill (Technion). Calf Intestinal Phosphatase (CIP) was purchased from New England Laboratories. ³⁵S-Methionine for *in vitro* and cell labeling was from Perkin Elmer. TNF α and the TNT® coupled transcription and translation kit were from Promega. Chir99021 and Ubiquitin were from Sigma-Aldrich. U1026 and PD98059 were from Cell Signaling (#9900). CVT-313 (SC-221445) and LY 2228820 were from SCBT.

Antibodies used in this study: Polyclonal and monoclonal (8D10) mouse α -RNF4 antibodies were generated by immunizing BALB/c BYJ Rb(8.12) 5BNR/J mice with 100 μ g His-RNF4²⁹⁻¹²⁹ and used at 1:500. Other RNF4 antibodies used were: Rabbit α -RNF4 antibodies (PA5-35170, Thermo scientific) and a gift from Jorma Palvimo (University of Eastern Finland). Mouse polyclonal α -Dgrn (1:500; Barry *et al*, 2011). α -Actin (1:2000, MP Biomedicals); α -FLAG M2 (1:1000, Sigma); α -HA (1:2000 Covance); α p-Ser62-c-Myc (#13748 Cell signaling Technology); α -Myc N-262 and C-33 (1:20, 1:100, Santa Cruz); rabbit α -I κ B- α C21 (1:1000, Delta Bio-labs); rabbit α -PGC1 α (1:1000), a kind gift from Daniel P. Kelly, Sanford-Burnham Medical Research Institute, Florida, USA). α Ubiquitin FK1 monoclonal antibody (1:1000, Biomol), α -c-Jun (#sc1694 1:500) and p-c-Jun (#sc822 1:1000) were from SCBT.

Primers used for qPCR:

RNF4 fwd: 5'-CCCGAGATCTCCTTGGGAAGC-3'

RNF4 rev: 5'-TCATCGTCACTGCTCACCAC-3'

Axin2 fwd: 5'-TTCGGATGTTTCATCCTGTGTTCCGG-3'

Axin2 rev: 5'-TGGTGCAAAGACATAGCCAGAACC-3'

GAPDH fwd: 5'-ACATCAAGAAGGTGGTGAAGCAGG-3'

GAPDH rev: 5'-AGCTTGACAAAGTGGTCGTTGAGG-3'

Plasmids: Human cDNA encoding RNF4 was cloned into pcDNA3 EcoRI-XhoI sites to generate pcDNA3 hRNF4. pcDNA3 RNF4^{C159A}, RNF4^{K179D}, RNF4 ^{Δ ARM}, and β -catenin^{S45A} were generated using the Quik-Change site-directed mutagenesis kit (Stratagene®) and fully sequenced. pcDNA3 constructs encoding rat RNF4 (rRNF4, rRNF4^{CS1}, and rRNF4 ^{Δ SIM}; Tatham *et al.*, 2009) were a kind gift from Ron T. Hay (University of Dundee). pcDNA3-HA-c-Myc, His-SUMO2 were kind gifts from Martin Eilers (Würzburg University). FLAG-ODC and FLAG-Az1 were generously provided by Chaim Kahana (Weizmann Institute). TOP-Luc, FOP-Luc, 6xMycTag- β -catenin, β -catenin^{S33A}, pcDNA3-N-Myc, and pBABE c-Myc were kind gifts from Mara Conacci-Sorrell, and Robert Eisenman (Fred Hutchinson Cancer Center). pRSET-Hairy, pcDNA3-TLE1, pcDNA3-HA-Max, pGL3-E-Box reporter, pcDNA3-pFlag-Fbw7 α , psPORT PGC1 α , 3xFLAG-NICD, pcDNA3, I κ B α and NF κ B-Luc reporter were as described (Welcker *et al.* 2004; Ong *et al.*, 2008; Trausch-Azar *et al.*, 2015). pCAGGS3.2-HA-ubiquitin and ubiquitin K>R mutants and K* were a gift of Kazuhiro Iwai (Kyoto University). p- cDNA His-c-Jun was a gift from Ami Aronheim (Technion-IIT).

Delta-Notch-CO-culture reporter assay:

Briefly, MK4 cells were seeded in 24-well plates at 6x10⁴ cells/ml and transfected the following day with 200ng TP1 canonical Notch reporter, 10ng CMV β -galactosidase and 50ng or 100ng pcDNA3 RNF4. The following day MK4 cells were co-cultured with CHO-IRES-GFP or CHO-Dll1-IRES-GFP cells at 1x10⁵ cells/ml, as indicated. Cells were harvested 36h post-transfection and luciferase and β -galactosidase activities were determined.

Generation of constitutive shRNF4 targeting and scrambled (sc) control (mission™):

shRNF4 C1:

5'-CCGGGACAAGCTCAGAAGCGAACTCCTCGAGGAGTTCGCTTCTGAGCTTGTCTTTTTG-3'

shRNF4 C2:

5'-CCGGCATCTGCATGGACGGATACTCCTCGAGGAGTATCCGTCCATGCAGATGTTTTG-3'

Scrambled shRNA:

5'-CCGGGCCCCAACTCGATAGAGAAGACTCGAGTCTTCTCTATCGAGTTGGGGCTTTTT-3'

Viral production and infection of constitutive shRNF4 and sc control: Lentiviral particles were generated using HEK293T cells transfected with the appropriate pLKO expression vectors and third-generation packaging vectors (pMission Sigma-Aldrich) using standard procedures. Infections were performed using standard protocol. In brief: for each infection, 1 \times 10⁶ cells were re-suspended in 1ml of virus-containing supernatant with 10 μ g/ml polybrene (American Bioanalytical, Natick, MA) in 24-well plates. Cells were then centrifuged at 140 \times g for 60min, re-suspended in culture medium, incubated for 16 h at 37°C, and transferred to culture dishes for an additional 48h. In rescue experiment, viral vectors coding for either c-Myc or GFP were co-expressed.

Generation of conditional Dox-induced shRNF4-targeting and Renilla control (miR-30-based):

Doxycycline miR-E stem loop based shRNAs targeting hRNF4 or Renilla (control) were generated from 97-mer oligonucleotides (BioTez), and cloned into the XhoI/EcoRI site of the lenti-viral pInducer11 construct (Fellmann, et al. 2013, Meereby., et a. 2011).

shRNF4-1: shRNA-ID: RNF4.1229 (TAATAGTGCTACATCTTGTGGG),

shRNF4-2: shRNA-ID: RNF4.2110 (TTTTCATTCAAACCTAACTGGAA),

shRenilla: Ren.713 as described in (Zuber et al. 2011; Fellmann, et al. 2013).

Respective Lentiviral particles from HEK293T cell supernatant were used to infect SW620 and MDA-MB-231 cell lines and passaged three times before FACS sorting for stable GFP expressing cells. After o/n induction with 200ng/ml Doxycycline, high responders were FACS sorted for dsRED and GFP and further cultured without Doxycycline. Stability of c-Myc protein was tested in SW620 and MDA-MB-231 stable and doxycycline-inducible control and shRNF4 cells. ShRNA expression was induced with 200ng/ml Doxycycline.

Generation of lenti-viral pInducer21-RNF4^{Dox} vectors: RNF4, RNF4^{C159A}, and RNF4^{ΔARM} were sub-cloned to pENTER GatewayTM vector and subsequently to pINDUCER21 lenti-viral Dox-regulated expression vector (Meereby et al., 2011). Inserts in destination vectors were verified by sequencing.

Supplemental References:

1. Bercovich, Z., Rosenberg-Hasson, Y., Ciechanover, A., Kahana, C. (1989). Degradation of ornithine decarboxylase in reticulocyte lysate is ATP-dependent but ubiquitin-independent. *J Biol Chem* 264: 15949-15952.
2. Fellmann et al. (2013) An optimized microRNA backbone for effective single-copy RNAi. *Cell Rep.* 5:1704-1713.
3. Gillette, T. G., Kumar, B., Thompson, D., Slaughter, C. A., DeMartino, G. N. (2008). Differential roles of the COOH termini of AAA subunits of PA700 (19S regulator) in asymmetric assembly and activation of the 26 S proteasome. *J. Biol. Chem.* 283:31813-31822.
4. Meereby., et al. (2011). The pINDUCER lentiviral toolkit for inducible RNA interference *in vitro* and *in vivo*. *PNAS USA.* 108: 3665-3670.
5. Tatham M. H., Rodriguez, M. S., Xirodimas, D. P., Hay, R. T. (2009). Detection of protein SUMOylation *in vivo*. *Nat Protoc* 4: 1363–1371.
6. Zuber J., McJunkin, K., Fellmann, C., Dow, L. E., Taylor, M. J., Hannon, G. J., Lowe, S. W. (2011) Toolkit for evaluating genes required for proliferation and survival using tetracycline-regulated RNAi. *Nat Biotechnol.* 29: 79-83.